UNIVERSITI PUTRA MALAYSIA

DEVELOPMENT OF A COMPETITIVE CHAIN REACTION ASSAY FOR QUANTITATIVE ANALYSIS OF WHITE SPOT SYNDROME VIRUS GENE TRANSCRIPTION AND VIRAL REPLICATION IN SHRIMPS

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FPV 2005 4

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By

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

February 2005

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

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February 2005

Chairman: **Professor Dato' Mohamed Shariff Mohamed Din, PhD**

Faculty: **Veterinary Medicine**

Despites much research on infectivity and diagnostics of white spot syndrome virus (WSSV), little is known about the viral replication kinetics and quantitative gene expressions. Therefore, a time course quantitative study was carried out using competitive polymerase chain reaction (cPCR) to measure viral growth in grow-out *Penaeus monodon* experimentally infected via feeding of WSSV infected tissue. The current tissue tropism studies demonstrated that gills have higher viral load followed by integument and abdominal muscle. Gills and integument were infected as early as 14 hour post infection (hr p.i.) compared to 24 hr p.i. for abdominal muscle. Gills are therefore recommended for extraction of DNA in routine PCR screening of WSSV. A classification of infection level was proposed to categorise infection into light (0 to 24 hr p.i.), moderate (24 to 48 hr p.i.) and moribund (48 to 120 hr p.i.) stage according to viral loads detected in

gills, which were 0 to 1×10^3, 1×10^3 to 1×10^7 and 1×10^7 to 1×10^9 copies per mg tissue respectively for the three infection stages. As the viral load was low at light infection, but increased exponentially at moderate infection and maintained at high level at moribund infection, such pattern of growth in viral loads is comparable to the eclipse, logarithmic and plateau phase of viral growth curve. White spots and reddish discoloration on the exoskeleton were apparent in moderate and moribund infection stage, but terminal clinical signs such as abnormal swimming behaviour and heavy mortality could only be observed in the later.

Previous studies on WSSV early genes expression were often qualitative rather than quantitative. By using competitive reverse transcriptase PCR (cRT-PCR), early gene ribonucleotide reductase large subunit (RR1) and thymidine kinase-thymidylate kinase (TK-TMK) mRNA expressions were non detectable at light infection stage (12 hr p.i.), but abundant at moderate (24 hr p.i.) and moribund (60 hr p.i. and above) infection stages. Geomeans of RR1 expression in whole heart samples were 9.69×10^4 and 2.36×10^7 copies at moderate and moribund infection stage respectively. Thus, both genes are probably vital in establishing WSSV infection, and their expressions are useful as marker in anti-viral studies of WSSV.

Shrimp immunity was emphasised under the European Commission's Shrimp Immunity and Disease Control (SI & DC) project. At present, prophenoloxidase (proPO) activating system and penaeidins, the predominant antimicrobial peptides, are well studied in bacterial and fungal infection, but not in viral infection. The mRNA expression of proPO was detected low and infrequent throughout infection with two-step PCR in heart and lymphoid organ. Penaeidin expression was however abundant with geomean of 4.35×10^4 copies in light infection (12 hr p.i.) but downregulated to 8.94×10^3 copies at moderate infection (24 hr p.i.) and non-detectable at moribund stage in whole heart samples. The lack of penaeidin and proPO mRNA upregulation suggests that they have little if any importance in the response to viral infection.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PEMBANGUNAN SATU ESEI REAKSI POLYMERAS BERANTAI KOMPETITIF UNTUK ANALISIS KUANTITATIF TRANSKRIPSI GENE DAN REPLIKASI VIRUS PENYAKIT SINDROM BINTIK PUTIH DALAM UDANG

Oleh

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Walaupun banyak kajian telah dibuat dalam jangkitan and diagnostik virus penyakit bintik putih (VPBP), kinetik replikasi virus dan expressi gen kuantitatif masih kurang diketahui. Oleh itu, kajian kuantitatif berpandukan masa telah dijalankan dengan menggunakan reaksi polymeras berantai kompetitif (cPCR) untuk menentukan pertumbuhan virus dalam udang *Penaeus monodon* yang dijangkiti VPBP dalam eksperimen dengan memberi makan tisu terjangkit VPBP. Kajian tropisma tisu ini menunjukkan bahawa insang mengandungi beban virus yang tertinggi diikuti oleh kulit dan otot abdomen. Insang dan kulit dijangkiti seawal 14 jam pasca infeksi (j p.i.) dibandingkan dengan 24 j p.i. untuk otot abdomen. Oleh sebab itu, adalah dicadangkan supaya insang digunakan untuk pengambilan DNA untuk pengujian PCR rutin untuk VPBP. Satu klasifikasi aras jangkitan diperkenalkan untuk mengkategori jangkitan kepada peringkat ringan (0

to 24 j p.i.), sederhana (24 to 48 j p.i.) dan berat (48 to 120 j p.i.) berpandukan beban virus yang dikesan dalam insang, yang masing-masing adalah 0 ke 1×10^3, 1×10^3 ke 1×10^7 dan 1×10^7 ke 1×10^9 salinan untuk ketiga-tiga peringkat jangkitan. Oleh kerana beban virus didapati rendah pada jangkitan ringan, tetapi bertambah secara eksponen pada jangkitan sederhana dan kekal pada paras tinggi semasa jangkitan berat, corak pertumbuhan beban virus boleh dibandingkan dengan fasa gerhana, logarithma and datar dalam keluk pertumbuhan virus. Bintik putih dan perubahan warna kemerahan pada rangka luar akan ketara dalam aras jangkitan sederhana dan berat, tetapi tanda-tanda klinikal terminal dan kematian serius cuma diperhatikan dalam yang terkemudian.

Kajian sebelum ini dalam ekspresi gen-gen awal VPBP lazimnya adalah secara kualitatif dan bukan kuantitatif. Dengan menggunakan transkriptas terbalik PCR kompetitif (cRT-PCR), ekpresi mRNA gen-gen awal virus seperti subunit besar reductase ribonucleotid (RR1) and kinase thimidin-kinase thimidilat (TK-TMK) tidak dapat dikesan dalam aras jangkitan ringan (12 j p.i.), tetapi tinggi pada aras jangkitan sederhana (24 j p.i.) dan berat (60 j p.i. dan ke atas). Min geometri ekspresi RR1 dalam sampel seluruh jantung adalah 9.69×10^4 dan 2.36×10^7 salinan masing-masing dalam aras jangkitan sederhana dan berat. Oleh itu, kedua-dua gen ini kemungkinan penting dalam pembentukan jangkitan VPBP dan ekspresi gen-gen berkenaan boleh diguna sebagai penanda dalam kajian anti-virus VPBP.

Kajian imuniti udang telah diutamakan di bawah projek Imuniti Udang Dan Kawalan Penyakit (SI & DC) Komisyen Eropah. Pasa masa kini, sistem pengaktifan prophenoloxidas (proPO) and penaeidin, sekumpulan peptid antimikrob dominan, telah dikaji secara terperinci dalam jangkitan bakteria dan fungi, tetapi bukan dalam jangkitan virus. Ekspresi mRNA proPO didapati rendah dan tidak kerap sepanjang jangkitan dengan pemeriksaan PCR dua langkah dalam jantung dan organ limfoid. Walaubagaimanapun, ekspresi penaeidin didapati tinggi dalam kedua-dua organ itu dengan min geometri setinggi 4.35×10^4 salinan pada aras jangkitan ringan (12 j p.i.) tetapi pengawalan menurun kepada 8.94×10^3 salinan pada aras jangkitan sederhana (24 j p.i.) dan tidak dapat dikesan pada aras jangkitan berat. Kekurangan peningkatan pengawalan ekspresi mRNA penaeidin dan proPO and penaeidin mencadangkan bahawa kedua-duanya adalah kurang penting dalam tindakbalas terhadap jangkitan WSSV.

ACKNOWLEDGEMENTS

First and foremost, I would like to say thanks to Prof. Dato' Dr. Mohamed Shariff bin Mohamed Din, my main supervisor who has given me this opportunity to pursuit my doctoral degree. His trust and confidence in my work are highly appreciated. The advice and guidance given throughout the course of studies are invaluable.

I am grateful to Assoc. Prof. Dr. Hassan bin Hj. Mohd. Daud who is my co-supervisor. Without his support and guidance, the course of my study would not be smooth.

I am also thankful to another co-supervisor, Assoc. Prof. Dr. Abdul Rahman Omar for providing constructive advices in molecular biology.

My thanks also go to senior PhD fellows Dr. Samson Soon, Dr. Wang Yin Geng and Dr. Abeer Hassan Sahtout who helped me during my early years of studies. Special thank goes to Dr. Lee Kok Leong, who is my friend and comrade for many years. Thanks also go to other colleagues at Aquatic Animal Health Unit namely Dr. Sanjoy Banerjee, Dr. Najiah Musa, Dr. Devaraja, Dr. Agus Sunarto, Miss Fennie Fong, Dr. Ng Chi Foon, Mr. Harry Anthony and Mrs. Wang. I am

also thankful to Faculty of Veterinary Medicine for providing supports during the course of study.

I am forever in debt to my parents, third and forth aunties, youngest uncle and siblings for their love and sacrifice. Lastly, I would like to thank Miss Kong Li-Lian for her companion, understanding, love and sacrifice.

I certify that an Examination Committee met on 17th February 2005 to conduct the final examination of Tan Lee Tung on his Doctor of Philosophy thesis entitled "Development of a Competitive polymerase Chain Reaction Assay for Quantitative Analysis of White Spot Syndrome Virus Gene Transcription and Virus Replication in Shrimps" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulation 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

TAN LEE TUNG

Date: 7 JUN 2005

TABLE OF CONTENTS

			Page
ABSTRACT			ii
ABSTRAK			v
ACKONWLEDGEMENTS			viii
APPROVAL			x
DECLARATION			xii
LIST OF TABLES			xvi
LIST OF FIGURES			xviii
LIST OF ABBREVIATIONS			xxii
CHAPTER			
1	GENERAL INTRODUCTION		1.1
2	LITERATURE REVIEW		
	2.1	Historical Background	2.1
	2.2	Classification and Nomenclature	2.2
	2.3	Morphology and Ultrastructure	2.4
	2.4	Pathogenesis and Epizootiology	2.5
	2.5	Molecular Biology Techniques for Diagnostics and Research of WSSV	2.9
	2.6	Genome Organisation and Replication	2.12
	2.7	Immune Mechanisms of Shrimp	2.13
3	DEVELOPMENT OF COMPETITIVE POLYMERASE CHAIN REACTION TECHNIQUE AS QUANTITATIVE ASSAY FOR WHITE SPOT SYNDROME VIRUS		
	3.1	Introduction	3.1
	3.2	Materials and Methods	3.6
		3.2.1 Primer Designs	3.6
		3.2.2 Construction of Competitive Template	3.7
		3.2.3 Cloning of Competitive Template	3.8
		3.2.4 Quantification of Competitive Template	3.12
		3.2.5 Validation of Assay Specificity, Sensitivity and Reproducibility	3.15
		3.2.6 Post-PCR Gel Densitometric Analysis and cPCR Assay Design	3.16
	3.3	Results	3.17
	3.4	Discussion	3.19

4 QUANTITATIVE STUDY OF WHITE SPOT SYNDROME VIRUS IN *PENAEUS MONODON* USING COMPETITIVE PCR
4.1 Introduction 4.1
4.2 Materials and Methods 4.3
4.2.1 Experimental Infection 4.3
4.2.2 DNA Extraction and Purification 4.4
4.2.3 Preliminary Analysis using Conventional Diagnostic Nested PCR 4.4
4.2.4 cPCR Assay 4.6
4.3 Results 4.7
4.4 Discussion 4.9

5 DETECTION OF EARLY GENES OF WHITE SPOT SYNDROME VIRUS USING DEGENERATE PRIMERS AND LOW-STRINGENT DNA HYBRIDISATION
5.1 Introduction 5.1
5.2 Materials and Methods 5.3
5.2.1 Identification of Conserved Region of Baculovirus Early Genes 5.3
5.2.2 PCR Amplification with Degenerate Primers 5.5
5.2.3 Construction of Gene Probes 5.6
5.5.4 Low Stringent Dot-blot DNA Hybridisation Assay 5.7
5.3 Results 5.11
5.4 Discussion 5.14

6 DEVELOPMENT OF COMPETITIVE REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION (cRT-PCR) ASSAY FOR QUANTITATIVE mRNA EXPRESSION STUDY OF VIRAL EARLY GENES AND HOST IMMUNE GENES
6.1 Introduction 6.1
6.2 Materials and Methods 6.4
6.2.1 Isolation of mRNA and Generation of Complimentary DNA (cDNA) 6.4
6.2.2 Construction of Competitive Templates 6.10
6.2.3 Validation of Competitive Templates 6.12
6.2.4 Post-PCR Gel Densitometric Analysis 6.13
6.2.5 cPCR Assay Mathematical Model and Design 6.13
6.3 Results 6.16
6.4 Discussion 6.17

7 QUANTITATIVE ANALYSIS OF VIRAL EARLY GENES AND HOST IMMUNE GENES mRNA EXPRESSION IN EXPERIMENTALLY INFECTED *PENAEUS MONODON* USING cRT-PCR.
7.1 Introduction 7.1
7.2 Materials and Methods 7.5
7.2.1 Experimental Infection 7.5
7.2.2 Total RNA Extraction 7.5
7.2.3 cRT-PCR Assay 7.7
7.3 Results 7.9
7.4 Discussion 7.11

8 GENERAL DISCUSSION AND CONCLUSION 8.1

REFERENCES R.1
APPENDICES A.1
BIODATA OF THE AUTHOR B.1

LIST OF TABLES

Table		Page
1.1	Commodities produced and traded (Export value, unit 1000US$).	1.8
1.2	World aquaculture production (metric tonne).	1.8
1.3	World aquaculture production (value: 1000 US$).	1.9
1.4	World aquaculture production for penaeid shrimp in 2001 (metric tonne).	1.10
3.1	Technical parameters of PCR primers.	3.22
3.2	Molecular sizes of native template (NT), competitive template (CT) and nested end point PCR template.	3.22
3.3	Concentration standard calculation for Low DNA Mass Ladder (Gibco BRL, USA) for concentration measurement of competitive template (CT).	3.22
3.4	Calculation of band intensity of competitive template (CT) and native template (NT) for cPCR analysis of WSSV-infected integument sample at 72 hr p.i.	3.23
4.1	Preliminary analysis on experimental WSSV infection with diagnostic nested PCR.	4.19
4.2	Viral load of experimentally infected *P. monodon* as determined by cPCR.	4.20
4.3	Geometric means of viral load from experimentally infected *P. monodon.*	4.20
4.4	Cumulative mortality in experimentally infected *P. monodon.*	4.20
4.5	Summary of quantitative analysis of experimental WSSV infection.	4.21
5.1	Technical parameters of degenerate PCR primers.	5.18

5.2	Technical parameters of PCR primers baculovirus immediate-early genes.	5.18
6.1	Technical parameters of PCR Primers.	6.24
6.2	Molecular sizes of Native template (NT) and competitive template (CT) of viral early genes, and *P. monodon* immune and housekeeping genes.	6.25
7.1	Viral load in gills and mRNA expression quantitations of viral early gene RR1 and host penaeidin gene in heart of experimentally infected *P. monodon.*	7.18
7.2	Viral early gene RR1 expression quantitation in various organs of moribund sample No. 5 as determined by cRT-PCR.	7.18
7.3	Quantification of penaeidin mRNA expression in heart of experimentally infected *P. monodon* using end point diluted cDNA amplified with two-step PCR, followed by Poisson analysis.	7.19

LIST OF FIGURES

Figure		Page
2.1	Average monthly rainfall in three geographical regions of Peninsular Malaysia based on Malaysian Meteorological Service, Ministry of Science, Technology and the Environment.	2.15
2.2	Seasonality of WSSV cases in east coast of Peninsular Malaysia based on diagnostic record at Universiti Putra Malaysia from 1997 to 2004.	2.16
2.3	Seasonality of WSSV cases in middle and southern west coast of Peninsular Malaysia based on diagnostic record at Universiti Putra Malaysia from 1997 to 2004.	2.16
2.4	General external anatomy of adult *Penaeus monodon*.	2.17
2.5	General internal anatomy of adult *Penaeus monodon*.	2.17
3.1	Synthesis and cloning of competitive template (CT).	3.24
3.2.	Estimation of CT concentration using mass ladder.	3.25
3.3	Quantification based on limiting dilution PCR method.	3.26
3.4.	Restriction digestion of PCR products with *Xba* I.	3.26
3.5.	Sensitivity of cPCR attributed to total number of PCR cycle.	3.27
3.6	Reproducibility of cPCR.	3.28
3.7	cPCR analysis of WSSV-infected integument sampled at 72 hr p.i.	3.29
4.1	Clinical signs of WSS in a *P. monodon* sample collected at 48 hr p.i.	4.22
4.2	Viral growth curve as determined by cPCR in *P. monodon* experimentally infected with WSSV.	4.23
4.3	cPCR gel images analysis and viral load quantification for experimental WSSV infection.	4.24

5.1	Optimisation of PCR programme for amplification of DNA polymerase gene.	5.18
5.2	Restriction fragment length polymorphism (RFLP) analysis of DNA polymerase gene PCR product.	5.19
5.3	PCR amplification of DNA polymerase gene from AcNPV and WSSV genomic DNA.	5.19
5.4	PCR amplification of *Artemia* actin and crayfish ribosomal protein gene with degenerate primer.	5.19
5.5	Gene probe constructions for immediate-early gene LEF-2 and IE-2.	5.20
5.6	Restriction fragment length polymorphism (RFLP) analysis of PCR product of immediate early gene IE-2.	5.20
5.7	Restriction fragment length polymorphism (RFLP) analysis of PCR product of LEF-2.	5.20
5.8	Flow chart of hybridisation and colourimetric detection in gene homology study.	5.21
5.9	Hybridisation with IE-2 probe at hybridisation temperature 68°C and 42°C.	5.22
5.10	Hybridisation with LEF-2 probe at hybridisation temperature 68°C and 42°C.	5.22
5.11	Hybridisation with IE-2 probe in 20% formamide hybridisation buffer.	5.23
5.12	Hybridisation with LEF-2 probe in 20% formamide hybridisation buffer.	5.23
5.13	Signal intensity comparison of hybridisation buffer using three different formamide concentrations.	5.23
5.14	Hybridisation using standard pBR328 DNA and probe to verify the effect of formamide on assay sensitivity.	5.24
5.15	Sensitivity comparison of colorimetric and chemiluminescent detection method.	5.24

6.1	Schematic diagram of mRNA isolation, cDNA synthesis and cDNA quality assessment with PCR.	6.26
6.2	cDNA quality assessment by PCR amplification with adaptor primer.	6.27
6.3	Reproducibility assessment of PCR amplification of with adaptor primer.	6.27
6.4	Schematic diagram of construction of competitive template (CT) based on Celi *et al.* (1993).	6.27
6.5	Two-step PCR amplification of WSSV early gene RR1, immune gene proPO and penaeidin from 10-fold diluted cDNA extracted from abdominal muscle of moribund shrimp infected with WSSV.	6.28
6.6	PCR amplification of WSSV early gene TK-TMK and shrimp actin genes from 10-fold diluted cDNA extracted from abdominal muscle of moribund shrimp infected with WSSV.	6.29
6.7	PCR amplification of shrimp GADPH gene from 10-fold diluted cDNA extracted from abdominal muscle of moribund shrimp infected with WSSV.	6.29
6.8	Purity assessment of TK-TMK competitive template (CT) purified by agarose gel electrophoresis and extracted by Qiaquick™ Gel Purification Kit (Qiagen, Germany).	6.30
6.9	Flow chart of quantification of competitive templates (CTs) of TK-TMK and RR1 using UV spectrometer and cPCR.	6.30
6.10	Quantification of WSSV genomic DNA as control to quantify CTs using cPCR.	6.31
6.11	Sensitivity assessment of cPCR of RR1, proPO and penaeidin.	6.31
6.12	Quantification of RR1 competitive template (CT) by cPCR co-amplified with pre-quantified WSSV genomic DNA control.	6.32
6.13	cRT-PCR analysis of RR1 gene expression in heart at 24 hr p.i.	6.33
6.14	Reproducibility assessment of cRT-PCR assay of RR1 gene expressions.	6.35

7.1	Anatomical location of lymphoid organ.	7.20
7.2	Screening for genomic DNA contamination using diagnostic nested PCR in total RNA and cDNA sample extracted from a WSSV-infected moribund shrimp.	7.20
7.3	GADPH gene transcription in heart and lymph organ as detected by RT-PCR.	7.21
7.4	Comparison of actin and GADPH gene expression in lymphoid organ as detected by RT-PCR.	7.21
7.5	mRNA expression of WSSV early gene RR1 and TK-TMK, and immune gene proPO and penaeidin as amplified by one-step RT-PCR.	7.22
7.6	mRNA expression of proPO as determined by two-step RT-PCR.	7.23
7.7	Numeration of haemocyte in heamolymph sample of moribund sample No. 5 in Neubauer counting chamber of haemacytometer.	7.23
7.8	mRNA expression of penaeidin in haemocyte, heart, lymphoid organ and gills (Lane 1 to 4 respectively) of a WSSV-infected *P. monodon* at moribund stage (Moribund No. 5).	7.24
7.9	Quantitative mRNA expression of viral early gene RR1 and immune gene penaeidin in heart at light, moderate and moribund WSSV infection.	7.24
7.10	Quantification of WSSV viral load in experimentally infected *P. monodon* using cPCR.	7.25
7.11	Quantification of RR1 mRNA expression in heart of experimentally infected *P. monodon* using cRT-PCR.	7.28
7.12	Quantification of penaeidin mRNA expression in heart of experimentally infected *P. monodon* using cRT-PCR.	7.30
7.13	Quantification of viral early gene RR1 mRNA expression in moribund sample No. 5 as determined by cRT-PCR.	7.31
7.14	Quantification of WSSV viral load (as target copy of RR1 gene) in gills of moribund sample No. 5 by cPCR.	7.33

LIST OF ABBREVIATIONS

AcNPV	*Autographa californica* nucleopolyhedrovirus
AMV	avian myeloblastosis virus
BSA	bovine serum albumin
BGBP	beta glucan binding protein
BMNV	baculoviral midgut necrosis virus
BP	*baculovirus penaei*
bp	base pair
CBV	Chinese baculovirus
cDNA	complementary deoxyribonucleic acid
cPCR	competitive PCR
CT	competitive template
CV	coefficient of variation
DNA	deoxyribonucleic Acid
dNTP	2'-deoxyribonucleoside 5'-triphosphate
DIG	digoxigenin
EST	expressed sequence tag
ELISA	enzyme-linked immunosorbent assay
EF-1α	eukaryotic elongation factor
FAO	Food and Agriculture Organisation of the United Nations
g	gram
g	gravitational force
GADPH	glyceraldehyde 3-phosphate dehydrogenase
geomean	geometric mean
HHNBV	hematopoietic necrosis baculovirus
HPV	hepatopancreatic parvovirus
H & E	haematoxylin and eosin
HzV-1	*Heliothis zea* virus 1
hr	hour
hr p.i.	hour post infection
ICTV	International Committee on Taxonomy of Viruses
IE-2	immediate early gene 2
IHHNV	infectious hypodermal and haematopoietic necrosis virus
IPTG	isopropyl-β-D-thiogalactoside
kD	kilo Dalton
LEF-2	late expression factor 2
L-DOPA	3-4 dihydroxyphenyl L alanine
LO	lymphoid organ
mg	milligram
min	minute
mL	millilitre
mM	millimolar

MBV	*Penaeus monodon*-type baculovirus
M-MLV	Moloney murine leukemia virus
mRNA	messenger ribonucleic acid
MW	molecular weight
µL	microlitre
µM	micromolar
NBT-BCIP	5-bromo-4-chloro-3-indoyl phosphate-nitroblue tetrazolium
NT	native template
ng	nanogram
OIE	Office International des Épizooties
oligo(dT)	Oligodeoxythymidine
ORF	open reading frame
OrV	*Oryctes rhinoceros* virus
OD	optical density
PCR	polymerase chain reaction
pfu	*Pyrococcus furiosus*
pmol	picomole
PRDV	penaeid rod-shaped DNA virus
proPO	propenoloxidase
REO	reo-like viruses
RFLP	restriction fragment length polymorphism
RT	reverse transcription
RR1	ribonucleotide reductase large subunit
rRNA	ribosomal ribonucleic acid
RV-PJ	rod-shaped nuclear virus of *Penaeus japonicus*
RT-PCR	reverse transcriptase polymerase chain reaction
s	second
SAPMP	streptavidin-paramagnetic particles
SEMBV	systemic ectodermal and mesodermal baculovirus
SDS	sodium dodecyl sulphate
SOI	severity of infection
SSC	standard sodium citrate
TE buffer	Tris-EDTA buffer
$TCID_{50}$	tissue culture infective dose
TK-TMK	thymidine kinase - thymidylate kinase
Tm	Melting temperature
UV	ultra violet
vWF	von Willebrand factor
WSBV	white spot syndrome baculovirus
WSS	white spot syndrome
WSSV	white spot syndrome virus
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

CHAPTER 1

1. GENERAL INTRODUCTION

Currently, penaeid shrimp farming is probably the most lucrative aquaculture venture. According to FAO Fisheries Department, frozen shrimps and prawns are the largest fishery commodity produced and traded internationally with a total export value of 7.5 billion US$ in 2001 (Table 1.1). Giant tiger prawn (*Penaeus monodon*), the major cultured shrimp species, ranked 19th in aquaculture production quantity in 2001, but the high value of this commodity at 4.7 billion US$ has rendered it No. 1 in terms of value (Table 1.2 & 1.3). Malaysia was the 11th top shrimp producer in the world with a production of 27,014 metric tonnes in 2001. Similar to many other countries in Asia, the major culture species in Malaysia is *P. monodon*, which is also the most cultured species worldwide contributing 48% to total shrimp aquaculture production in 2001 (Table 1.4). Shrimp culture still relies heavily on wild brooders as source of post larvae and therefore constantly exposed to the risk of disease introduction. To prevent this, good disease screening facilities using sensitive molecular biology based detection techniques have to be implemented. Western Hemisphere on the other hand, has the option of culturing domesticated specific pathogen free (SPF) shrimp species which is free from major shrimp pathogens. But culturing of such